

# Glutamic acid 160 is the acid-base catalyst of $\beta$ -xylosidase from *Bacillus stearothermophilus* T-6: a family 39 glycoside hydrolase

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**Abstract** A  $\beta$ -xylosidase from *Bacillus stearothermophilus* T-6 was cloned, overexpressed in *Escherichia coli* and purified to homogeneity. Based on sequence alignment, the enzyme belongs to family 39 glycoside hydrolases, which itself forms part of the wider GH-A clan. The conserved Glu160 was proposed as the acid-base catalyst. An E160A mutant was constructed and subjected to steady state and pre-steady state kinetic analysis together with azide rescue and pH activity profiles. The observed results support the assignment of Glu160 as the acid-base catalytic residue. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

**Key words:**  $\beta$ -Xylosidase; Glycoside hydrolase family 39; Acid-base catalyst; Mechanism; *Bacillus stearothermophilus*

## 1. Introduction

Xylans, the most abundant hemicelluloses of the plant cell wall, are composed of  $\beta$ -1,4-linked D-xylopyranosyl backbone substituted with glucuronosyl, arabinosyl and acetyl side chains. Because of its complex structure, the complete degradation of xylan requires the synergistic action of several hemicellulolytic enzymes [1,2]. Among others, these include endo-1,4- $\beta$ -xylanases (EC 3.2.1.8), which hydrolyze the xylan backbone, and  $\beta$ -xylosidases (EC 3.2.1.37), which cleave the resulting xylooligomers to free xylose. Interest in xylan-degrading enzymes stems from their potential applications in the paper and pulp industry for biobleaching and for the bioconversion of lignocellulose material to fermentative products [3,4]. Recently, these enzymes, as well as other glycosidases, were demonstrated as potential glycosynthases for oligosaccharide synthesis [5].

Glycoside hydrolases cleave the glycosidic bond either by retention or inversion of the anomeric configuration of the

substrate [6]. Inverting glycosidases use a single-displacement mechanism with the assistance of a general acid and a general base [7]. Retaining glycosidases follow a two-step double-displacement mechanism involving two key active site residues, one functioning as the nucleophile and the other as the acid-base [7].

To date,  $\beta$ -xylosidases are assigned to glycoside hydrolase families 39, 43 and 52 based on sequence similarities [8,9]. The glycoside hydrolase family 39, which itself is part of the wider GH-A clan, was shown to cleave the glycosidic bond with retention of the anomeric configuration [10]. All families belonging to clan GH-A were found to share conserved structural motifs that allow, together with sequence alignment, the prediction of the two catalytic residues [11]. Using this information, the catalytic pair of glycoside hydrolases family 39 was predicted, and recently the nucleophile of this family was identified in the *Thermoanaerobacterium saccharolyticum*  $\beta$ -xylosidase using 2-deoxy-2-fluoro- $\beta$ -D-xylopyranoside as a mechanism-based inactivator [12]. The catalytic acid-base residue of this family is yet to be identified.

Family 39 also contains other enzymes, the  $\alpha$ -iduronidases, which are involved in the lysosomal degradation of glycosaminoglycans in higher organisms [13]. Deficiencies in these enzymes lead to accumulation of partially degraded glycosaminoglycans, resulting in mucopolysaccharidosis type I known as the Hurler or Scheie syndromes [14].

As part of our ongoing effort to study the hemicellulolytic system in *Bacillus stearothermophilus* T-6, we have recently cloned and sequenced a 23.5 kb chromosomal segment that contains a cluster of xylan utilization genes [15].

This region includes a  $\beta$ -xylosidase gene (*xynB1*) showing homology to family 39 glycoside hydrolases. The *xynB1* gene is part of an operon responsible for the intake and catabolism of aldotauronic acid (2-O- $\alpha$ -(4-O-methyl- $\alpha$ -D-glucuronosyl)-xylotriose) formed by the primary degradation of xylan [15]. We describe here the overexpression, purification and biochemical characterization of XynB1 from *B. stearothermophilus* T-6, and provide firm support for the assignment of Glu160 as the acid-base catalyst of family 39.

## 2. Materials and methods

### 2.1. Cloning of the *xynB2* gene

Based on the DNA sequence of the *xynB1* gene (AF098273) from

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**Abbreviations:** PNXP, *p*-nitrophenyl  $\beta$ -D-xylopyranoside; ONPX, *o*-nitrophenyl  $\beta$ -D-xylopyranoside; 2,5-DNPX, 2',5'-dinitrophenyl  $\beta$ -D-xylopyranoside; 3,4-DNPX, 3',4'-dinitrophenyl  $\beta$ -D-xylopyranoside; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

*B. stearotherophilus* T-6, two PCR primers that allow the in-frame cloning of the gene into the T7 polymerase expression vector pET9d (Novagen), were designed. The N-terminal primer (5'-GGAATTC-CATGGGTGTTGTAAACGTGCCAAGCAA-3') included an ATG translational start codon inside an *Nco*I restriction site (CCATGG). The C-terminal primer (5'-CGGGATCCTTCATGAAGAATACGA-TGTTATTTACC-3') contained a stop codon (TAG) and a *Bam*HI restriction site (GGATCC) after the end of the gene. Following amplification, the PCR product was purified, digested with *Nco*I and *Bam*HI and cloned into *Nco*I/*Bam*HI-digested pET9d vector, resulting in the plasmid pET9d-*xynB1*.

## 2.2. Protein production and purification

*Escherichia coli* BL21(DE3) pET9d-*xynB1* was grown overnight on Terrific Broth [16] with kanamycin (25 µg ml<sup>-1</sup>) without induction (4×500 ml in 2 l shake flasks shaken at 230 rpm and 310 K). The overnight culture was harvested, resuspended in buffer (60 ml of 50 mM Tris-HCl and 100 mM NaCl pH 7.0) and disrupted by two passages through a French press at room temperature. The cell extract was centrifuged and the soluble fraction was heat treated (333 K, 30 min) and then centrifuged again at room temperature. The enzyme was further purified by gel filtration at room temperature on a Superdex 200 26/10 column (Pharmacia) running at 2.5 ml min<sup>-1</sup> (50 mM Tris-HCl buffer pH 7.0, 100 mM NaCl and 0.02% NaN<sub>3</sub>). Enzyme purity was estimated by SDS-PAGE [17] and by gel filtration. This purification procedure resulted in about 100 mg of nearly homogeneous enzyme from 1 l culture, with an overall yield of 60% and 8-fold purification.

## 2.3. Kinetic studies

The substrates 2',5'-dinitrophenyl β-D-xylopyranoside (2,5-DNPX) and 3',4'-dinitrophenyl β-D-xylopyranoside (3,4-DNPX) were synthesized as described by Ziser et al. [18]. *p*-Nitrophenyl β-D-xylopyranoside (PNPX) and *o*-nitrophenyl β-D-xylopyranoside (ONPX) were obtained from Sigma. All kinetic studies were carried out either continuously or by stopped assay conditions. Continuous reactions were performed at 40°C in a 1 ml cuvette. Assay solutions contained 600 µl of appropriately diluted enzyme with different substrate concentrations ranging from 0.1 to 5 *K<sub>m</sub>*, in 100 mM phosphate buffer (pH 7.0). The increase in absorbance was followed using a Biochrom 4060 spectrophotometer (Pharmacia). The extinction coefficients used and wavelength monitored for each substrate were as follows: 4'-nitrophenyl, 420 nm, Δε=7.61 mM<sup>-1</sup> cm<sup>-1</sup>; 2'-nitrophenyl, 420 nm, Δε=1.91 mM<sup>-1</sup> cm<sup>-1</sup>; 2',5'-dinitrophenyl, 420 nm, Δε=3.68 mM<sup>-1</sup> cm<sup>-1</sup>; 3',4'-dinitrophenyl, 400 nm, Δε=11.15 mM<sup>-1</sup> cm<sup>-1</sup>. Values of *K<sub>m</sub>* and *k<sub>cat</sub>* were determined by non-linear regression analysis using the program GraFit 3.0 [19]. Pre-steady state reactions were performed similarly, with 2,5-DNPX at 0.2°C (this temperature was obtained using a methanol water bath) (Δε=2.80 mM<sup>-1</sup> cm<sup>-1</sup>) and PNPX at 40°C.

Stopped assay reactions were used for pH dependence studies. Reactions were carried out in 12 ml glass tubes (13×100 mm) placed in a water bath at 50°C with PNPX as a substrate. The mixtures contained 600 µl of 5 mM substrate and appropriately diluted enzyme in 100 mM of the appropriate buffer. The buffers used were: citric acid-Na<sub>2</sub>HPO<sub>4</sub> (pH 4.0–6.5), phosphate buffer (pH 6.0–8.0) and Tris-HCl buffer (pH 7.5–9.0). The reactions were stopped by the addition of Na<sub>2</sub>CO<sub>3</sub> (final concentration of 0.3 M), and the released *p*-nitrophenyl was determined spectrophotometrically using a 1 ml cuvette and an extinction coefficient of Δε=18 mM<sup>-1</sup> cm<sup>-1</sup>.

## 2.4. Mutagenesis

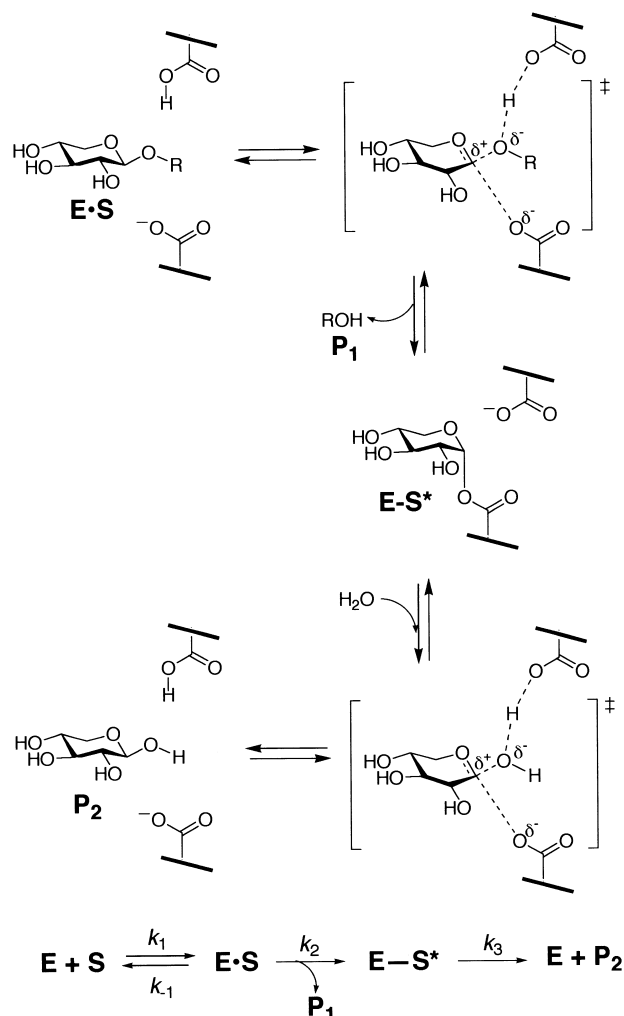
Mutagenesis was performed directly on the pET9d-*xynB1* double-strand DNA following the method of Meza et al. [20]. This strategy employs four oligonucleotides in two sequential PCR reactions, using two flanking primers (the N-terminal and C-terminal primers that were used for cloning the *xynB1* gene) and two different internal primers, one of which was the mutagenic primer that was designed to introduce a unique restriction site (*Bsm*I) enabling the detection of the desired mutation. The C-terminal primer together with the mutagenic primer, and the N-terminal together with the internal primer, were used to amplify two overlapping fragments that were subsequently annealed and elongated. The flanking primers (described previously) were then added to amplify the entire mutated *xynB1* gene. The mutagenic and internal primers were the following: E160A, 5'-

CACTAAATTAGGTGCATTCCATACTTC-3'; internal, 5'-GGCG-TGATCTCATCGTAGCGG-3'. The mutated nucleotides are in bold and the restriction site is underlined. The mutation was confirmed by DNA sequencing.

## 3. Results and discussion

### 3.1. Sequence analysis of *XynB1*

The *xynB1* gene from *B. stearotherophilus* T-6 was cloned, efficiently overexpressed in *E. coli* and purified to homogeneity. The amino acid sequence of the protein was scanned with the BLAST2 [21] program and showed 66, 65%, 65, 64, 37 and 33% identity to β-xylosidases from *Bacillus halodurans* (BAB04787), *Thermoanaerobacterium* sp. (AAB68820), *T. saccharolyticum* (AAA27369), *Caldicellulosiruptor saccharolyticus* (AAB87373), *C. saccharolyticus* (AAA23063) and *Aeromonas caviae* (BAA95685), respectively. It also showed 31, 28 and 22% identity to three α-iduronidases from *Homo sapiens* (AAA51698), *Canis familiaris* (AAA51455) and *Mus musculus* (AAC42044), respectively, and 33 and 25% identity to two ORFs from *Pseudomonas aeruginosa* (AAG05625) and *Drosophila melanogaster* (AAF53070), respectively. All these proteins have been classified to family 39 in the general classification of the glycoside hydrolases [8,9].



Scheme 1. Proposed mechanistic pathway for retaining glycosidases.

Table 1  
Kinetic parameters for hydrolysis of aryl- $\beta$ -D-xylosides by XynB1 and its E160A mutant

Phenol substituent	pK <sub>a</sub>	Enzyme	$k_{\text{cat}}$ (s <sup>-1</sup> )	$K_{\text{m}}$ (mM)	$k_{\text{cat}}/K_{\text{m}}$ (s <sup>-1</sup> mM <sup>-1</sup> )
2,5-Dinitro	5.15	WT	8.91	0.95	9.38
		E160A	0.097	0.016	6.04
3,4-Dinitro	5.36	WT	9.0	0.8	11.24
		E160A	0.1	0.025	4.25
4-Nitro	7.18	WT	8.22	5.2	1.58
		E160A	0.0044	0.27	0.02
2-Nitro	7.22	WT	20.88	7.1	2.94
		E160A	0.0079	0.18	0.04

### 3.2. Identification of the acid-base catalyst

Amino acid alignment of family 39 glycosidases revealed a highly conserved sequence WNEP. This sequence is a conserved motif in clan GH-A [11], implicating the glutamic acid in this sequence, Glu160, as the putative acid-base in XynB1. To verify the role of Glu160, this amino acid was substituted with alanine and the kinetic properties of the mutant E160A were determined.

Catalysis by clan GH-A members follows the well accepted mechanistic pathway shown in Scheme 1 [6]. This is a two-step double-displacement mechanism involving the formation of a covalent enzyme-bound intermediate E-S\*. The two chemical steps undertaken at the enzyme active site include: a glycosylation step, in which the enzyme's nucleophilic residue, usually Glu or Asp, is glycosylated with the sugar substrate; a deglycosylation step, in which the above glycosyl-enzyme intermediate undergoes hydrolysis to reconstitute free enzyme [11]. During the glycosylation step, the enzymatic acid-base catalytic residue is assumed to protonate the glycosidic bond oxygen of the substrate, thereby facilitating bond cleavage by stabilizing the leaving group (P<sub>1</sub>). During this event, the enzymatic nucleophile attacks the anomeric carbon of the sugar substrate to form a covalent glycosyl-enzyme intermediate (E-S\*). In the deglycosylation step, the carboxylate of the acid-base catalytic group serves as a general base to activate an incoming water molecule, which attacks the anomeric carbon of the sugar in the glycosyl-enzyme intermediate to form free sugar product (P<sub>2</sub>) and reconstituted enzyme, and thus accomplishing the catalytic cycle [11].

According to the above mechanistic scheme, substitution of the acid-base catalytic residue by mutation with other non-acidic residues should affect the rates of both chemical steps, though the effect on each step will be different. The effect on the glycosylation step will depend strongly on the leaving group ability of the aglycone. Rates of hydrolysis for substrates with a poor leaving group should be affected much strongly than those with a good leaving group. As such, if we assume that the glycosylation step is rate limiting then the  $k_{\text{cat}}$  values will vary with substrate reactivity. The deglycosylation step, however, will be affected equally for all substrates carrying different leaving groups because the same glycosyl-enzyme intermediate is hydrolyzed during this step [22]. Thus, if we assume that the deglycosylation step is rate limiting, then the  $k_{\text{cat}}$  values should be invariant for all substrates carrying different leaving groups.

**3.2.1. Catalysis with substrates bearing different leaving groups.** In attempts to test the effect of the acid-base replacement, the kinetic constants of the native enzyme and the E160A mutant were determined using substrates with different leaving groups (Table 1).

Hydrolysis of all four substrates by the native enzyme resulted in similar  $k_{\text{cat}}$  values indicating that the second step, deglycosylation, is the rate limiting step since it is independent of substrate reactivity. As expected, the  $k_{\text{cat}}$  values measured for the E160A mutant were significantly reduced with all substrates used, distinguishing Glu160 as a catalytic residue. Interestingly, this reduction in rate constant was only 90-fold for both 3,4-DNPX and 2,5-DNPX (substrates with good leaving groups) and  $1.8 \times 10^3$ - and  $2.6 \times 10^3$ -fold for PNXP and

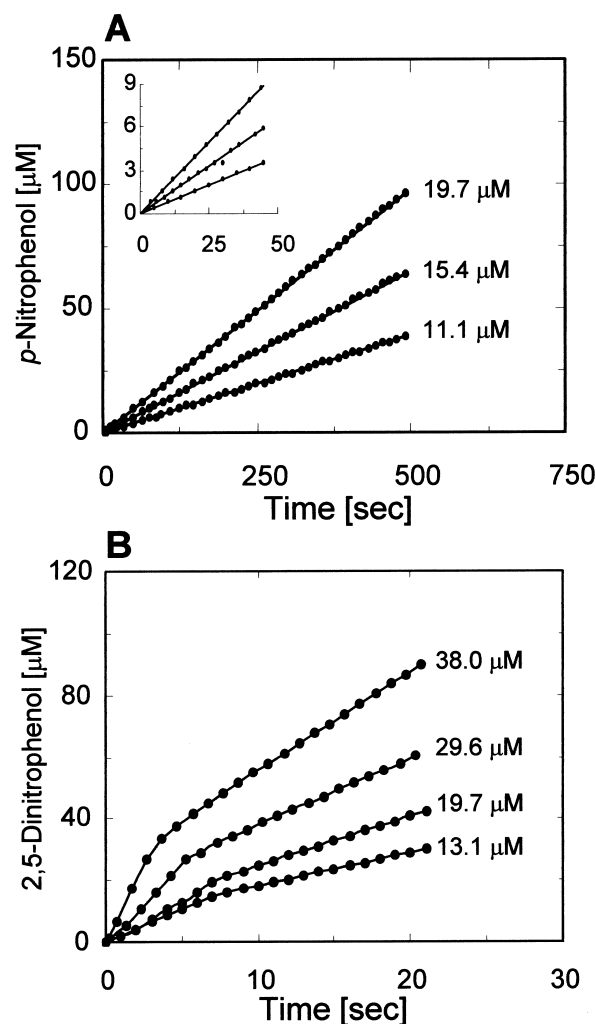


Fig. 1. Pre-steady state kinetics for the hydrolysis of (A) PNXP and (B) 2,5-DNPX by XynB1 E160A. Enzyme concentrations are indicated for the individual curves. All reaction mixtures contained 0.6 mM substrate. The inset shows the same traces over a shorter period of time.

ONPX (substrates with poor leaving groups), respectively. Since the  $k_{\text{cat}}$  values of the E160A mutant vary with substrate reactivity, it is obvious that the rate limiting step is different for some of the substrates. Thus, the first step became rate limiting, obviously for substrates having a poor leaving group (PNPX and ONPX) since their phenolic group needs more protonic assistance for departure, while the second step is probably rate limiting for the substrates with the good leaving group (2,5-DNPX and 3,4-DNPX). Furthermore, hydrolysis of 2,5-DNPX and 3,4-DNPX by the mutant resulted in very low  $K_m$  values (Table 1), suggesting that a glycosyl–enzyme intermediate accumulates [7] as would be expected if the second step is rate limiting. The consequence is that the E160A mutation changed the rate limiting step from deglycosylation step (for the wild-type) to glycosylation (for the E160A mutant) for substrates that require a strong acid assistance.

**3.2.2. Burst kinetics.** If indeed the first step is rate limiting for substrates with poor leaving groups ( $\text{p}K_a < 5.5$ ), and the second step for substrates with good leaving groups, then a pre-steady state burst should exist only for the latter substrates. Detection of such a burst was feasible using a standard spectrophotometer since the reaction rates by the E160A mutant at 0.2°C were sufficiently slow. As seen in Fig. 1A, hydrolysis of PNPX by the mutant showed no pre-steady state burst kinetic indicating that the first step is rate limiting. Conversely, with 2,5-DNPX as a substrate (Fig. 1B), a burst phase proportional to enzyme concentration was observed, indicating that for this substrate the second step is rate limiting.

**3.2.3.  $k_{\text{cat}}/K_m$  values.** Further assessment of the effect of the acid-base mutation on the glycosylation step can be obtained by comparison of the  $k_{\text{cat}}/K_m$  values of the wild-type and the E160A mutant (Table 1) [22]. These values were relatively similar for both the wild-type and the E160A mutant for hydrolysis of 2,5-DNPX and 3,4-DNPX. However, the  $k_{\text{cat}}/K_m$  values of the E160A mutant were two orders of magnitude smaller than those of the wild-type for hydrolysis of PNPX and ONPX. Thus, elimination of Glu160 had little effect on the rates of the first step for hydrolysis of substrates with good leaving groups that require little or no acid assistance. However, the rates of the first step were severely affected for hydrolysis of substrates with poor leaving groups that require strong acid assistance. This result gives additional support for the assignment of Glu160 as the acid-base catalyst.

**3.2.4. Azide rescue.** The role of Glu160 as the acid-base catalyst can also be examined by the use of external nucleophilic anions such as azide [22]. In this procedure, coined ‘azide rescue’, the small azide anion enters the vacant place created by the alanine replacement, and reacts with the anomeric carbon instead of a water molecule to form the corresponding  $\beta$ -glycosyl azide product [23]. In the absence of an acid-base catalyst, which normally provides general base catalysis during the second step, the deglycosylation step is severely affected. Addition of these external anions in many cases accelerates the second step, therefore increasing  $k_{\text{cat}}$  (if the second step is rate limiting). Indeed, in the presence of 2 M azide, the  $k_{\text{cat}}$  values increased about 8-fold for the hydrolysis of 2,5-DNPX by E160A (Fig. 2A). As a consequence of the acceleration of the second step, the  $K_m$  values also increased, since less glycosyl–enzyme intermediate accumulated [7]. The net result is that the  $k_{\text{cat}}/K_m$ , which reflect the first step, remained relatively constant, suggesting that the

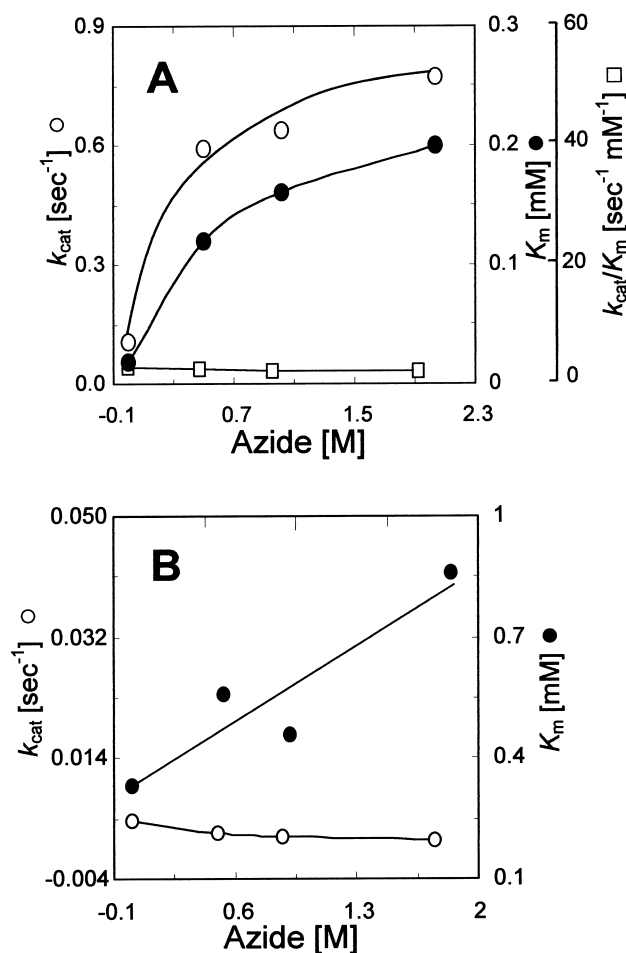


Fig. 2. Kinetic parameters for hydrolysis of 2,5-DNPX (A) and PNPX (B) by XynBI E160A in the presence of various azide concentrations.

presence of azide had no effect on the glycosylation step. In contrast, with PNPX as a substrate the presence of azide did not increase  $k_{\text{cat}}$  (Fig. 2B), suggesting that the first step is rate limiting. This result is another indication that the first step is rate limiting for the hydrolysis of poor substrates by E160A. In addition, since the rate of the second step is accelerated, and the rate of the first step is probably unchanged, less glycosyl–enzyme intermediate accumulates resulting in the increase of  $K_m$  values (Fig. 2B).

**3.2.5. pH profiles of the wild-type and the E160A mutant.** The assignment of Glu160 as the acid/base catalyst can also be tested by looking at the pH dependence profiles for the wild-type and the E160A mutant using PNPX as substrate (Fig. 3).

As expected, the pH dependence profile of the native enzyme is a typical bell-shape curve, reflecting the ionization of the two active site carboxylic acids. However, with the E160A mutant no reduction in activity was observed at high pH values suggesting that the protonated group (acid catalyst) had been removed. Interestingly, the pH dependence curve of the mutant was shifted, and the  $\text{p}K_a$  of the acidic limb, which reflects the nucleophile, was elevated in approximately 2 pH units. It was previously suggested that the ionized nucleophile, distanced approximately 0.5 nm from the acid-base

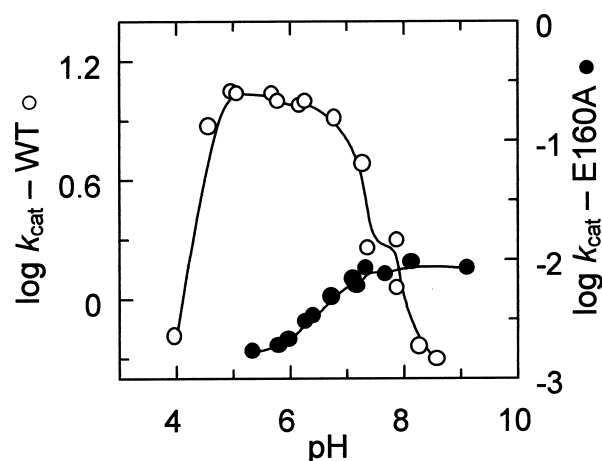


Fig. 3. pH dependence for the hydrolysis of PNPX by wild-type XynB1 and its E160A mutant.

in retaining glycosidases, elevates the  $pK_a$  value of the acid-base due to charge repulsion [24]. The replacement of the acid-base probably affected, directly or indirectly, the ionization state of the nucleophile, thereby elevating its  $pK_a$  and reducing its acidity, thus, shifting higher the pH optimum. Determination of activity at pH values higher than 10 was precluded since the enzyme precipitated.

In conclusion, taking together the results provide unequivocal confirmation for the earlier prediction of Glu160 as the acid-base catalyst in XynB1, thereby removing any uncertainty for the assignment of this residue in glycoside hydrolase family 39.

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